Profiles of Insulin-Like Growth Factor Binding Proteins and the Protease Activity in the Maternal Circulation and Its Local Regulation Between Placenta and Decidua

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Abstract. Insulin-like growth factors (IGF) and their specific binding proteins (IGFBPs) are believed to be important regulators of fetal growth. IGFBP protease which proteolyses IGFBPs and changes the biochemical properties of IGFBPs is also presumed to be involved in fetal growth. The aim of this study is to elucidate the physiological significance of IGFBP protease in fetal growth and regulators of protease in placenta and decidua. The intact IGFBP-3 was proteolyzed into fragments when pregnant serum was incubated with $^{125}$I-IGFBP-3. IGFBP-3 protease activity showed a marked increase at 5 weeks of gestation and reached a plateau in maternal circulation at 15 weeks of gestation. These changes in protease activity correlated with the profiles of IGFBPs in the maternal circulation analyzed by Western ligand blot, where the IGFBP-1 is only the dominant IGFBP. The intact IGFBP-3 was proteolyzed when culture media of decidual cells were incubated with $^{125}$I-IGFBP-3, but was not proteolyzed when culture media of trophoblast cells were incubated with $^{125}$I-IGFBP-3. Decidual protease activity was slightly increased by IGF-I and completely inhibited by progesterone. The protease activity was more increased in the mothers with growth retarded infant than in those in the mothers with normal growth infants, suggesting that the protease activity is elevated in compensation for the impaired fetal growth. These results suggest that increased protease activity in maternal blood may be involved in the fetal growth indirectly by reducing the binding activity of IGFBP-3 to IGF-I, and that protease activity in maternal blood may be derived from decidua that is regulated by placental hormones.

Key words: Insulin-like growth factor (IGF), IGF binding protein (IGFBP), Protease, Pregnancy, Fetal growth

INSULIN-LIKE growth factor (IGF) is one of the growth factors. Two types of polypeptides with similar structures are known: IGF-I and IGF-II [1, 2]. Most IGF in the blood and humoral systems is found as a complex with IGF binding proteins (IGFBPs). Six different IGFBPs have been reported, and IGFBP-1 to 6 [3], and their amino acid sequences have been identified. IGFBP-3 is most commonly found in the blood [4]. Since the affinity of IGFBP-3 for IGF is higher than that of other IGFBPs [5], approximately 80% of IGF is found to be bound to IGFBP-3 [4]. IGFBPs are known to either stimulate [6, 7] or suppress [8, 9] the action of IGF. Maternal IGF-I was shown to stimulate fetal growth by increasing the transfer nutrients to the fetus through the placenta [10], and fetal IGF-I is presumed to stimulate fetal growth by promoting anabolic events and DNA synthesis in fetal tissues [11]. In contrast, many studies have indicated that IGFBP-1 inhibits the biological action of IGF-I [8]. Recently it was reported that IGFBPs are cleaved by specific proteases [12, 13]. Once IGFBPs are cleaved by proteases, their binding affinity for IGF decreases [14], resulting in a change in its effect on IGF [15, 16].
IGF-II in the maternal circulation is on the same level as in the non-pregnant period, but IGF-I increases throughout the progress of gestation and is positively correlated with fetal growth [17]. On the other hand, the activity of specific proteases on IGFBPs in the maternal circulation also increases [18, 19]. Increased protease activity modifies the profiles of IGFBPs during the gestational period compared to those in the non-pregnant period. Four IGFBPs, IGFBP-1, -2, -3 and -4, have been identified in the circulation by Western ligand blot during the non-pregnant period [20], but the binding activity of IGFBP-2, -3, and -4 was found to be remarkably suppressed at the beginning of the gestational period by pregnancy-associated protease [18, 19]. IGFBP-1 is not affected by protease and increases throughout the gestational period, and its level is inversely correlated with birth weight [21]. IGF-I and IGFBP-1 therefore play central roles in the IGF-IGFBPs system during pregnancy, and the balance of these two is thought to be an important factor in regulating fetal growth. Several IGFBPs are produced by the uterine decidua [22]; particularly IGFBP-1 is secreted in large amounts in relation to placental progesterone [23]. This uterine decidual production is presumed to be the origin of the increase in IGFBP-1 in maternal blood.

In this study, we analyzed the profiles of IGFBPs protease activity in the maternal circulation, and attempted to determine its physiological role in fetal growth. We also examined whether or not the trophoblast and decidua produce proteases, and discuss the related control mechanisms.

**Materials and Methods**

**Materials**

$^{125}$I-IGF-I, and $^{125}$I-IGF-II were purchased from Amersham Life Science (Tokyo, Japan). $^{125}$I-IGFBP-3 was purchased from Diagnostic System Laboratories Inc. (Webster, Texas, USA). Collagenase (type I), DNase-I, progesterone and 17β-estradiol were purchased from Wako-Junyaku-Kogyo (Osaka, Japan). Hank’s Balanced Salt Solution was purchased from Gibco Co. (Grand Island, New York, USA). Nitrocellulose trans-blot membranes were purchased from Bio-Rad Laboratories (Richmond, Virginia, USA).

**Blood samples**

Blood samples were collected from 5 non-pregnant women with normal menstrual cycles. The blood was drawn from the cubital vein at the beginning of the follicular phase. Maternal blood samples were collected from 40 normal pregnant women without maternal complications at various gestational weeks and from 14 women at puerperium days 1 to 5. The blood samples were centrifuged to collect the serum. The serum was frozen at $-80 \, ^\circ C$ until protease activity was measured. The serum from the 5 non-pregnant women was pooled but the samples from the pregnant and puerperal women were kept separately. Gestational weeks were calculated from the last menstrual period and reevaluated by measuring crown-rump length at 10 weeks of gestation. Maternal blood was also collected from 10 mothers with a normal growing fetus and 7 mothers with an intrauterine growth retarded fetus (−1.5 SD below mean weight) at 30 to 34 weeks of gestation. All these IUGR fetuses were asymmetrical IUGR due to uteroplacental insufficiency and none of the IUGR cases selected for this study were chromosomal disorder and multiple pregnancy.

**Western ligand blot**

Western ligand blot was performed according to the method of Hossenlopp [20]. Ten microliters of maternal serum was analyzed by SDS polyacrylamide gel electrophoresis (12.5% polyacrylamide) without reduction, and protein was transferred onto nitrocellulose membrane in a Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol). The nitrocellulose membrane was then incubated in 10 mM Tris-HCl (pH 7.4, 1% BSA, 0.1% Tween-20) including $^{125}$I-IGF-I (600,000 cpm) and $^{125}$I-IGF-II (400,000 cpm) at 4 °C for 24 h. After incubation, the membrane was washed with 10 mM Tris-HCl (pH 7.4, 1% BSA, 0.1% Tween-20), and autoradiography was carried out for seven days by exposing the X-ray film (X-OMT AR Film, Eastman Kodak Co., Rochester, New York, USA) to the membrane.
**Cultures of decidua and trophoblast cells**

The full-term placenta in clean condition was washed in cold phosphate-buffered saline (1.0 mM, pH 7.4) including 1 mM EDTA before the decidua and trophoblast tissues were isolated. The decidua and trophoblast tissues were sliced and incubated with collagenase (10 mg/g tissue) and DNase-I (1 mg/g tissue) in medium 199 (Gibco Co., Grand Island, New York, USA) at 37 °C for 30 min to separate the cells. After the cells were filtered through nylon mesh to remove undigested tissue, the culture solution was centriﬁuged at 800 × g for 10 min. Hematocytes were eliminated by recentrifuging the collected cells in 60% Percoll (Pharmacia Fine Chem., Uppsala, Sweden). Isolated cells were cultured in medium 199 (25 mM HEPES, 2.2 g/l NaHCO₃, 10% fetal calf serum, 1% penicillin/streptomycin, pH 7.4) with 95% air and 5% CO₂ for 48 h. The medium was changed to Hank's Balanced Salt Solution (2.2 g/l NaHCO₃, pH 7.4), incubated for 24 h, and the supernatant was then collected. Cells induced from the decidua were cultured either with or without IGF-I (100 nmol/l), 17β-estradiol (1 μg/ml), or progesterone (10 μg/ml) for 24 h. The supernatant was ultraﬁltered to an approximately one-tenth volume in a Centricon-10 Microconcentrator (MW cut off 10,000, Amicon Inc., Beverly, Massachusetts, USA) before performing the protease assay. Progesterone and 17β-estradiol were dissolved in ethanol, but the final concentration of ethanol in the cell culture was controlled to 1 μl/ml.

**Protease assay**

Protease activity in the serum and in the culture supernatants of the decidua and trophoblast cells was measured according to Lamson et al. [24]. 125I-IGFBP-3 (30,000 cpm) was incubated either with the serum (5 μl) or culture supernatant (50 μl) at 37 °C for 5 h. IGFBP-3 in the reaction solution was separated from the digested products by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide), and the dried gel was used for autoradiography. Absorbance of radioactive bands on an X-ray film for 125I-IGFBP-3 and its digestion products was measured in a densitometer (CS-900, Shimazu Chemical Co. Ltd., Tokyo). The amount of proteolysis was determined as the percentage of the proteolytic cleavage products over the total density in each lane.

**Statistical analysis**

The effect of each agent on protease activity is shown as the mean ± SEM for 4 cultured medium samples. The significance of differences between maternal serum and cultured medium in protease activity was tested by Student's t-test.

**Results**

**Profiles of IGFBP and protease activity in maternal blood**

IGFBP-3, -2, -1 and -4, in that order of MW, were all identiﬁed in the blood of non-pregnant women (Fig. 1), but the binding activity of IGFBP-3 and -4 had completely diminished by 10 weeks of gestation, and that of IGFBP-2 was reduced. The binding activity of these IGFBPs had nearly recovered by puerperium day 1, and had completely recovered by day 5. On the other hand, the activity of IGFBP-1 increased during the gestational period. When 125I-IGFBP-3 was incubated with the sera of either non-pregnant or full-term pregnant women, cleavage of IGFBP-3 into several fragments was observed in the serum of pregnant but it was not in that of non-pregnant women (Fig. 2). Protease activity, indicated by the degradation of IGFBP-3, showed a rapid increase after 5 weeks of gestation, and reached a plateau by 15 weeks of gestation, where more than 60% of the 125I-IGFBP-3 was degraded. This level was maintained until the end of gestation (Fig. 3). This serum protease activity, however, decreased by puerperium day 1 and had completely disappeared on day 5.

The results of maternal protease assays for normal fetuses and intrauterine growth retarded fetuses are shown in Table 1. There are no differences between the two groups in maternal age or in gestational weeks, but the estimated weight of the fetuses was 1764.5 ± 162.8 g (n=10, mean ± SEM) for the normal group and 1136.7 ± 124.9 g (n=7) for the intrauterine growth retarded group, which was a significant difference (P<0.05).
Maternal serum protease activity in the normal fetus group was 71.5 ± 6.8%, but that in the intrauterine growth retarded group was 94.4 ± 5.2%, a significant difference (P<0.05).

Protease activity in placental trophoblast and decidual cells

Protease activity in cultured media of trophoblast and decidual cells from the full-term placenta was assayed after incubating with $^{125}$I-IGFBP-3 at 37 °C for 5 h. Although no degradation of IGFBP-3 was observed in the trophoblast cell medium incubation, the cultured medium of decidual cells produced several fragments of IGFBP-3, as in the incubation with maternal serum, and the cleavage pattern also showed similarities (Fig. 4). The decidual cells were further cultured with 100 nmol/l IGF-I, 10 µg/ml progesterone, or 1 µg/ml 17β-estradiol. The protease activity in the control medium was 60.5 ± 3.3%. The level of activity rose slightly (P<0.05) to 74.6 ± 4.6% with the addition of IGF-I, was remarkably suppressed (P<0.0005) to 5.4 ± 0.6% with progesterone, and did not change with 17β-estradiol (Table 2).
Since the degradation of IGFBP-3 by maternal serum shows maximum activity at 37 °C and a neutral pH, the reaction has been presumed to be catalyzed by an enzyme [18, 19]. The enzyme was considered to be a metal ion dependent serine protease because the reaction was inhibited by various serine protease inhibitors and EDTA [18, 19]. In this study, the protease activity was estimated by the degree of IGFBP-3 degradation. The protease activity correlated well with the binding activity profiles of IGFBP-3 in the blood of pregnant and puerperal women as determined by Western ligand blot. The binding activity of IGFBP-3 to IGF in maternal serum therefore seems to decrease in accordance with cleavage by this protease. The reaction of IGFBPs to protease differs depending on the type of binding protein; the changes in the binding ability of IGFBP-4 during the gestation and puerperal periods as detected by Western ligand blot were similar to those of IGFBP-3. In contrast, IGFBP-2 retained its binding ability even at 10 weeks of gestation, and IGFBP-1 was not at all affected by protease. Besides protease for IGFBP-3, several different proteases, for example, protease for IGFBP-2 [25], for IGFBP-4 [26], and for IGFBP-5 [27], have been reported. These are all metal ion dependent serine proteases,

![Fig. 3. Protease activity estimated by the degradation of 125I-IGFBP-3 in sera from women at various gestational weeks and postpuerperium.](image)

**Table 1.** Maternal protease activity in normal growth group (NG) and intrauterine growth retardation group (IUGR)

<table>
<thead>
<tr>
<th>Maternal age (y)</th>
<th>Gestational week (wk)</th>
<th>Estimated fetal body weight (g)</th>
<th>Protease activity (%)</th>
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<tr>
<td>NG (n=10)</td>
<td>32.1 ± 3.5</td>
<td>32.4 ± 2.6</td>
<td>71.5 ± 6.8</td>
</tr>
<tr>
<td>IUGR (n=7)</td>
<td>31.4 ± 4.8</td>
<td>31.3 ± 3.4</td>
<td>94.4 ± 5.2</td>
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</table>

*125I-IGFBP-3 was incubated with serum (5 μl) at 37 °C for 5 h. Degradative products of IGFBP-3 were separated by SDS-PAGE and detected by autoradiography.

**Discussion**

Since the degradation of IGFBP-3 by maternal serum shows maximum activity at 37 °C and a neutral pH, the reaction has been presumed to be catalyzed by an enzyme [18, 19]. The enzyme was considered to be a metal ion dependent serine protease because the reaction was inhibited by various serine protease inhibitors and EDTA [18, 19]. In this study, the protease activity was estimated by the degree of IGFBP-3 degradation. The protease activity correlated well with the binding activity profiles of IGFBP-3 in the blood of pregnant and puerperal women as determined by Western ligand blot. The binding activity of IGFBP-3 to IGF in maternal serum therefore seems to decrease in accordance with cleavage by this protease. The reaction of IGFBPs to protease differs depending on the type of binding protein; the changes in the binding ability of IGFBP-4 during the gestation and puerperal periods as detected by Western ligand blot were similar to those of IGFBP-3. In contrast, IGFBP-2 retained its binding ability even at 10 weeks of gestation, and IGFBP-1 was not at all affected by protease. Besides protease for IGFBP-3, several different proteases, for example, protease for IGFBP-2 [25], for IGFBP-4 [26], and for IGFBP-5 [27], have been reported. These are all metal ion dependent serine proteases,
but cleave each IGFBP specifically. It is therefore considered that several types of protease exist, but it remains unclear how many of them, whether one or more, are responsible for the increase in protease activity in the maternal blood. We have evaluated the protease activity in pregnancy sera by the degradation of $^{125}$I-IGFBP-4 and observed similar profiles to those by IGFBP-3 (data not shown). But it is difficult to estimate the precise protease activity for each IGFBP in sera since the possibility that one IGFBP protease in sera may proteolyze another IGFBP cannot be excluded.

The level of IGF-I in maternal blood rises during the gestational period [17]. This increase is seen not only in the total amount of IGF-I, but also in the free IGF-I which is not bound to IGFBPs. Cleavage of IGFBP-3 by protease produces a 30 kDa fragment with a reduced affinity for IGF [28]. That is the reason why IGFBP-3 in pregnancy serum is not detected by Western ligand blot, but a 30 kDa fragment still binds to IGF with less affinity for IGF-I than for IGF-II and the dissociation of IGF bound to the 30 kDa fragment is faster than intact IGFBP-3 [14]. Since approximately 80% of IGF-I is bound to IGFBP-3 [4], these changes in the biochemical properties of IGFBP-3 would be largely related to increases in free IGF-I in maternal blood.

Fetal growth before 20 weeks of gestation is relatively stable and is not being affected by maternal and fetal disorders except genetic disorders. The IGF system including IGF-I, IGFBP-1 and protease does not seemed to be involved in fetal growth in this period. The fetus shows remarkable development between 24 and 34 weeks of gestation and fetal growth in this period is largely dependent on the maternal nutritional supply. Maternal IGF-I and IGFBP-1 influence fetal growth by controlling the nutritional supply to the fetus through the placenta in this period [10]. As demonstrated in this study, IGFBP-1 is not affected by protease and increases throughout the gestational period, and its level is inversely correlated with birth weight [21]. Maternal IGFBP-1 suppresses fetal growth presumably by inhibiting the binding of IGF-I to placental receptors [10]. This suggests that IGFBP-1 is an important negative regulator of fetal growth during pregnancy. IGFBP-1 levels are high in both the mother and fetus with intrauterine growth retardation [21, 29]. In the rat, uterine artery ligation [30] and maternal starvation [31] that cause intrauterine growth retardation [21, 29]. In the rat, uterine artery ligation [30] and maternal starvation [31] that cause intrauterine growth retardation stimulate the production of IGFBP-1 by the fetal liver, the main production site of IGFBP-1. Taken together, the increase in IGFBP-1 may play a crucial role by which fetuses survive by reducing the consumption of nutrients and inhibiting their growth induced by IGF-I adapting.

### Table 2. Effect of IGF-I, 17β-estradiol and progesterone on protease activity in cultured media of decidual cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IGF-I (100 nmol/l)</th>
<th>17β-estradiol (1 µg/ml)</th>
<th>Progesterone (10 µg/ml)</th>
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<tbody>
<tr>
<td>Protease activity (%)</td>
<td>60.5 ± 3.3</td>
<td>74.6 ± 4.6*</td>
<td>63.8 ± 5.3</td>
<td>5.4 ± 0.6**</td>
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* $P<0.05$ vs. control, ** $P<0.0005$ vs. control.
themselves to poor environments such as uteroplacental insufficiency. Increased protease activity in maternal blood in cases of retarded intrauterine growth also supports this hypothesis. Proteolysis of IGFBP-3 increases not only free IGF-I in the circulation but also alters the distribution of IGFBP-3 among IGFBPs in the circulation. Approximately 80% of IGF circulates as a 150 kDa complex that consists of IGF-I or IGF-II plus IGFBP-3 and an acid labile subunit (ALS) in serum [4]. Although a proteolyzed 30 kDa fragment of IGFBP-3 can form the 150 kDa ternary complex in the presence of ALS [32], the distribution of IGF-I shifts from the 150 kDa complex to the small IGF-I-IGFBP complex [14]. As observed in this study, the binding capacity of IGFBP-4 is reduced to almost the same extent as IGFBP-3, and IGFBP-2 to a lesser degree, by pregnancy-associated protease, but IGFBP-1 remains intact and increases during pregnancy. Furthermore, IGFBP-1 has an affinity for IGF-I that is three times higher than that of proteolyzed IGFBP-3, but that for IGF-II is lower [4]. In these conditions, a considerable amount of IGF-I is presumed to shift from the 150 kDa ternary complex to the IGF-I-IGFBP-1 binary complex during pregnancy. In this way, increased protease activity in the maternal circulation stimulates the shift of the control of IGF-I activity by IGFBP-3 in nonpregnant status to that by IGFBP-1 during pregnancy. Circulatory IGFBP-3 levels are relatively stable [33] and considered to be storage site for IGF in the circulation. In contrast, IGFBP-1 levels are more acutely changed reflecting to nutritional factors [34]. Fetal growth is dependent on the maternal nutritional status, and maternal metabolism is more catabolic than that in the nonpregnant status to provide nutrition for the fetus. In these conditions, control of IGF-I action by IGFBP-1 is more physiological than control by IGFBP-3, since it is necessary to control IGFBP-3 activity quickly to correspond to the maternal nutritional status during pregnancy.

IGFBP-1 in the circulation is increased in mothers with intrauterine growth retardation compared to those in mothers with normal growing fetuses [21], and protease activity is increased in these mothers as demonstrated in this study. It is therefore possible that binding of IGF-I to IGFBP-1 is increased in mothers with intrauterine growth retardation and IGF-I activity is more suppressed in these mothers, thereby inhibiting fetal growth. Increased protease activity observed in mothers with intrauterine growth retardation may therefore play a role in enhancing the suppressive effect of IGFBP-1 on IGF-I action.

IGFBP-1 is secreted from the decidua in large amounts [35] and this appears to be the original production site for maternal IGFBP-1. Decidual IGFBP-1 production is stimulated by progesterone [23] and inhibited by IGF-I [36]. Since placenta produces not only progesterone but also IGF-I [37], it is evident that IGFBP-1 in the decidua is regulated by placentals hormones as well as IGFBP-1. Besides IGFBP-1, the decidua produces IGFBP-2 and IGFBP-4 [22]. These binding proteins suppress IGF-I activity [4] and cancellation of the suppressive activity of these IGFBPs by decidual protease [4] enhances local IGF-I activity to stimulate fetal growth. From this point of view, placental progesterone locally suppresses IGF-I activity at the placenta by increasing decidual IGFBP-1 and by suppressing protease activity that proteolyzes not only IGFBP-3 but also IGFBP-2 and IGFBP-4.

The IGFBP-3 cleavage pattern of the decidual protease was the same as that of maternal serum. This suggests that the decidua might be a possible production site for the protease that increases in the maternal blood but, increased protease activity in maternal circulation cannot be explained by the control of progesterone, since progesterone increases IGFBP-1 production by decidua but inhibits protease activity. In contrast, both IGFBP-1 levels and protease activity are increased in mothers with intrauterine growth retardation. Furthermore, no relation was observed between the maternal circulatory progesterone levels and fetal growth. Increased protease activity for IGFBP-3 in the circulation has been demonstrated after a surgical operation [38] and with diabetes mellitus [39] suggesting that multiple production sites have to be considered for circulatory protease activity during pregnancy although production sites other than decidua have not yet been elucidated.

In any case, the stimulation of fetal growth by
IGF-I depends largely on the placental/decidual local regulation system. Elucidation of this complicated regulation system would be of great help in further understanding the maternal-fetal interaction mechanisms between the placenta (fetal organ) and the decidua (maternal organ).

References


